REGENERATIVE POTENCIES OF DISSOCIATED CELLS OF HYDROMEDUSÆ.

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INTRODUCTORY.

At various times during my earlier work on the development and regeneration of hydromedusæ, especially that dealing with the developmental capacity of egg fragments, there had grown the conviction of the remarkable potencies of the various tissue elements of these organisms. So strong had this impression become that the desirability of repeating Trembly's picturesque experiment of turning *Hydras* inside out and testing again the possibilities that ectoderm and entoderm might really exchange functions under the new conditions involved was entertained. While admitting the rather convincing results of the experiments of Ischikawa ('90), touching this feature it still seemed that there might be some warrant that under certain conditions Trembly's conclusions might find confirmation. However, the writer never found the convenient season for trying out the experiment, though other correlated features were observed at various times in connection with the work above cited, especially in the summer of 1908 while working on the development of *Clava* and *Hydractinia*. This was further incited by the work of H. V. Wilson, "On Some Phenomena of Regeneration in Sponges," '07. Therefore with the opportunity for investigation at the Naples Laboratory I set about a series of experiments with a view to settle some of the problems concerned. My work at Naples began in December, 1910, and continued till mid-April following, and during this period systematic experiments were made upon the regenerative potencies of somatic cells of about a dozen different species, among which the following may be named: two species of *Eudendrium*, two species of *Tubularia*, one each of *Hydractinia*, *Podocoryne*, *Campanularia*, *Obelia*, *Halecium*, *Sertularia*, and a medusa, *Liriope exigua*.

In *Science* of March 10, 1911, appeared a preliminary report

of similar experiments by Dr. Wilson, which reached Naples about April 1, just when my own experiments were being concluded, results of which had already been written and some of which will appear in the following sections just as originally prepared. I immediately wrote Professor Wilson, giving a brief account of my work and stating that my results would be held pending the appearance of his completed report. This appeared in due course ('11). While dealing in the main with different material yet he had employed essentially the same methods which I had followed and they seemed so conclusive that I had laid my own paper aside, deeming it unnecessary. However, the report in the *Journal* of the Marine Biological Association of similar experiments by DeMorgan and Drew (Oct., '14) which seemed to express some doubt as to the conclusiveness of Wilson's results, prompts me to submit even at this late date my own results, though in somewhat abbreviated form.

Material and Methods.

Concerning material employed in the experiments mention has been made in the previous section as to the several species used, though two species of medusa instead of one were experimented upon. For the most part particular care was taken to have perfectly fresh and vigorous specimens, but it was later found that this precaution was not absolutely essential in all cases, some of my best hydranths having been reared from material which had been several days in the laboratory before being used. Later mention will also be made of a probable reason why this may happen. One point however calls for special emphasis, namely, that of the freshness and purity of the water used in the experiments. In my work several expedients were employed to guard against the presence of parasitic organisms, especially predatory protozoa. Of most efficiency was that of having water fresh from the open sea. Another expedient was that of sterilizing water of the aquaria, and still a third was that of using synthetic, that is, artificial sea-water. But of all these the first was found to be most satisfactory.

Concerning methods much might be said, though only the briefest reference will be made of those employed by me. Among

the several modes of isolating the tissue cells the following were employed: With hydroids the cœnosarc may be forced out of the perisarc by clipping off the hydranths and then stripping the stems through clean fingers, or the ends of smooth forceps, or similar device. Again, one may finely clip up the stems with scissors and then still further reduce the cells by continuing the operation in deep watch glasses or small beakers. A still further mode was used, that of clipping up the stems with scissors and later grinding the tissues under a smooth glass rod whose end had been rounded in the flame, employing it as a pestle and a watch glass as a mortar. One objection to this was the crushing of the individual cells in many instances, and otherwise injuring them. On the other hand there may be reason to believe that such treatment is not really so serious an injury as might at first sight appear, for as will be seen in some later discussion, the shock may actually serve as a stimulus to hasten cellular de-specialization and hence initiate regenerative processes.

Cell dissociation having been effected the next method is to arrange them in dishes where aggregation may take place. First in this operation is the straining or filtering out of debris and such fragments as are undesirable. This was done chiefly by pressing the cells through bolting cloth. In my experiments it was found that a better medium than silk bolting cloth was a fine meshed cheese cloth, or a coarse meshed linen or cotton fabric, which was softer and apparently more efficient. In a few cases in the earlier experiments I merely placed the entire mass in watch glasses and with a pipette carefully drew off most of the coarser stuff and left the cells as free as such a process might leave them. On the whole, the pressing process worked better and was more expeditious. Following this operation the cells were left for a time to settle and then the milky sediment was carefully drawn off, when fresh water was added and the dishes set aside in bowls surrounded by running water to ensure as constant a temperature as could be had.

The Experiments.

Podocoryne carnea.—This was the first species which came to hand and proved one of the most responsive and convincing

of the entire series. Several colonies were brought in, all occupying shells inhabited by hermit crabs, the usual habitat of this species. In one respect the species is rather difficult to operate on owing to the spinous condition of the basal cœnosarc, which made it hard to obtain enough of the polyps to make the desired culture. By allowing the colony to expand fully in shallow dishes it was possible by a dextrous sweep of the scissors to cut off quite a bunch at a single time, and by allowing others to expand in the same way and repeat the operation it was found possible to secure sufficient material for the culture. The specimens were finely cut or ground into a pulpy mass, filtered through the sterilized cloth, and thus fitted to undergo later changes. It may as well be stated here that among hundreds of preparations relatively few gave completely successful results in the regeneration of new polyps. My first surprise was not that many of the preparations "went bad," but that any survived the operation and went forward in regeneration. Here as in most experiments on regeneration a large mortality occurs in the preparations.

Character of the Dissociated Cells.—If examined soon after their dissociation one may easily distinguish the several sorts of cells even under a magnification of three hundred diameters, that is, ectoderm, entoderm, nematocyst, interstitial, etc. The very minute ectoderm cells are in striking contrast with the large flagellated cells of the entoderm. In the course of an hour, sometimes less, these differences become less marked, and ultimately almost disappear. They have become despecialized into potentially embryonic cells, and probably from this change have acquired their regenerative capacities. A careful study of such dissociated cells from various species has strongly suggested the probability that some such cytomorphic process is involved in most regenerative phenomena, and leaves little doubt that the features under consideration here are positively brought about through such a process.

Cell Aggregation.—Examination of a culture within a few hours, three to five, will show that a remarkable change has taken place among the cells in their relations to each other. They will be found to have formed numerous small nodular groups

having the appearance in many cases of embryonic morulæ, or blastulæ. It was this phenomenon among others already mentioned which first raised the question in my mind as to their regenerative possibilities many years ago. Concerning the mode by which this process of aggregation is brought about there is some doubt. The attempt was made to actually observe it by carefully keeping a fresh culture under direct observation with the microscope. It was thought that the action of the flagellated cells of the entoderm might act as a means by causing vortices in the water, but careful study failed to show that this was a factor of any direct value. Such action of these cells may be easily seen but its effects are as often repellent as attractive. The fact that certain of the cells show amœba-like aspects suggested a possible amœboid action in the process. But here again no evidence whatever was found to prove the suggestion. One might imagine some chemotropic influence, but no evidence was found that such was the case. I am inclined to the view that chance contact is perhaps the chief factor in the process. This is made probable by the fact that such aggregation may be greatly facilitated by mechanical agitation of the cells, and by a gentle rotary motion of the dishes. In the earlier experiments considerable care was taken to handle the dishes as little as possible during the early stages of an experiment, thinking such might be undesirable, but later the opposite view was taken, and the dishes often rotated to hasten the process. It must be admitted, however, that there seemed to be other factors involved, for even when a considerable mass of cells had been brought together by this means there was later found to have been a sort of segregative process at work, for the mass had been more or less broken up into sections or lobes which later behaved as entirely independent bodies.

The cell aggregates, while rather predominantly sub-spherical in shape, showed considerable variation. Some were flattish, or disk-like, and some were somewhat lobulated and irregular in shape. But throughout a series of such aggregates one of the most conspicuous features was that already referred to above, namely, the resemblance to an embryonic blastula or morula, especially a hydroid morula; and if one were to take account of

such morulæ as those of *Pennaria* or *Turritopsis* or *Hydractinia* it would include practically the entire range of shape exhibited by these regeneration aggregates, and one might designate them as regeneration morulæ, for such they really seem to be.

Encystment.—Following the process of aggregation there occurred in those vitally active a process of encystment, that is, the secretion of a definite perisarc about the entire mass, and its adhesion to the bottom of the glasses. Lest this feature be regarded as peculiar to these particular cases it should be pointed out that the phenomenon is often shown at a certain stage in the normal development of the hydromedusæ, and indeed in some scyphomedusæ as well. The writer has directed attention to this in the case of *Cyanea* ('02, '10) and it is doubtless shared by many others. Its function is doubtless protective, just as is that of the perisarc in the adult hydroid. Encystment usually occurs shortly following the completion of the phase of aggregation just described. This encysted stage may continue for an indefinite time, or it may be of short duration. The latter was more frequently the case with *Podocoryne* than with some others. In the present case the cyst was frequently ruptured for the upgrowth of the hydranth within a comparatively short time, say two days; but in many cases this stage persisted for a week or even more, and indeed in certain cases the cyst became a prison, being so dense as to become impenetrable from within as well as without. This again is comparable with what may happen in such stages in normal development (vide supra). This process of perisarc formation often takes various forms, following the phases of growth. In *Podocoryne* there was frequently the development of a reticulated hydrorhiza before the appearance of a hydranth, and later there appeared nodular enlargements of these stolon-like tubes and from these points would occur the upgrowth of a series of polyps. In one such preparation I obtained three vigorous young hydranths.

What has been stated in this connection as to *Podocoryne* is likewise true of other species experimented with. The behavior of the encysted aggregation morulæ is quite like that of the growing stolons of the hydrorhiza. Both may live for weeks under these conditions without any signs of further development.

Again, after such prolonged periods there may come about another direction of regenerative activity and a hydranth may arise. Aside from the evidence of life observed in the active circulation within the cœnosarc of stolons it becomes easy for one to recognize in the character of the cells of the various structures the evidences of life or death, and furthermore death of any portion is rapidly followed by disintegration brought about by microörganisms. It should have been stated in the earlier part of this section that the process of encystment usually begins soon after the aggregation phase is complete, which may be within twelve to twenty hours, though it may not become evident until much later, thirty to forty hours. The first evidence of its formation is the adhesion of the mass to the bottom of the glass, and somewhat later may be distinguished as a very delicate transparent film covering the entire mass. Its later extension may be easily followed as the growth of stolons takes place, which may be quite rapid in some cases, or in others very slow. Here again as was pointed out in an earlier connection, there is a marked similarity in the aspects of regenerative growth and those of embryonic development which further emphasizes the probability that they are fundamentally identical, having their initiative in potentially embryonic cells.

Polyp Formation.—In *Podocoryne* the first evidence of definitive hydranth organization was found during the second day following the experiment. This consisted in the dissolution of the cyst at its upper surface and the protrusion of a bud-like upgrowth. At first these were barely distinguishable, but during the third day they had become large enough to be seen with the unaided eye. The first fully formed hydranth appeared early on the fifth day, when a polyp having the distinctive form of hypostome and three tentacles was noted. This was followed by further growth of the young specimen in all its parts. The movement of the tentacles and their growth in length was interesting and striking, leaving not the least doubt as to the genuineness of the regenerative process. Usually the first three tentacles appeared at about the same time, but in a few cases it was noted that when first observed there were but two, though a third appeared rather soon after. The full six tentacles of the new polyp were

developed within the next two or three days, and conformed exactly to the phases of the growth of an embryonic specimen. It should be emphasized in this connection that in the rate of growth in these specimens, as in the entire regenerative process, there was great individual difference. Apparently this was dependent upon the state of vitality of the underlying organization. For example, it was found that development was slower, and the resulting polyps smaller when arising from small cell aggregates, and in cases where there had been an excessive stolonization prior to polyp formation. In the one case it would seem as if the store of energy was small to begin with, and in the second that it had been depleted by excessive stolon formation.

The young polyps continued to live for several weeks, much longer than would have seemed probable when the highly artificial conditions, and the very limited food supply are taken into consideration. During the course of the experiments more than a dozen of these polyps of *Podocoryne* were reared to functional maturity and many others to such stage as to leave no trace of doubt as to the validity of the results.

Let it be remarked here that in this species all the material was in the asexual condition, that is, there were polyps only, no signs whatever of medusæ, which are the sexual stage in the life cycle of *Podocoryne*. Other experiments go to show that so far as use of material of asexual or mixed condition no difference as to regenerative potency could be distinguished. In *Eudendrium* where the medusa stage is absent, and where one finds sex cells in various stages of growth, the experiments were apparently not thereby influenced at all. Indeed, in those cases in which egg cells were present they took no part whatever in later regenerative activity, either degenerating or being absorbed as yolk material.

Eudendrium.—In experiments upon *Eudendrium* two species were used, *E. rameum*, and *E. racemosum*, both very common at Naples. Methods of treatment were the same as in the case already described. The promptness with which these hydroids had responded in the numerous previous experiments by the writer[1] and others in regeneration and regulation led me to

[1] Biol. Bull., Vol. I., p. 35.

anticipate that a similar type of reaction might be anticipated in this connection, but as will be seen this expectation was not realized fully. The early reactions in aggregation, encystment, etc., were quite as prompt and promising as in *Podocoryne*. And in these features the species showed nothing peculiar. But beyond the initial stages the results were disappointing. The mortality was much greater and the growth reactions much less energetic. Experiments were varied in every way practicable, hydranths alone being used for obtaining disorganized cells, cœnosarc alone, male colonies alone and female colonies alone. There seemed to be no very marked differences in results, though the cells obtained from crushing hydranths gave the least satisfactory results. As already stated the early stages followed quite as in *Pocodoryne*, encystment, and stolonization, but beyond these my experiments were far less satisfactory than in the former. In only a few cases was I able to obtain polyps, and these were small and very weak. A few developed tentacles, but never the usual number, nor were they more than buds on the base of the hydranth. The few polyps which developed secreted the usual perisarc, which was indistinguishable from that of an embryonic *Eudendrium*.

Tubularia.—As in the former I employed two species, *T. mesembryanthemum*, and *T. larynx*. As in the former the early reactions were prompt and quite like the others. But unlike the others my experiments never afforded a single polyp. The massing of dissociated cells was quite as prompt and the resulting morula-like embryo as promising as in either of the others. The encystment of perisarc followed in due order, and these lived for many days, but they never showed further signs of development. Perhaps no hydroid genus has had so large a place in experimental work as has *Tubularia*. If therefore my anticipations as to the behavior in cellular regeneration of *Eudendrium* were disappointing, those concerning *Tubularia* were really perplexing, at least for the time being. I think an explanation may be ventured which, though not absolutely convincing, may relieve a measure of the perplexity. It is to the effect that regenerative potency in an organism is more or less conditioned by its state of vitality, or in still more suggestive phrase, its *physiological*

state, at the time it is subjected to the test. This has been recognized in principle in experiments on ordinary regenerative processes, in that only especially vigorous specimens are used. Further discussion of this point will be deferred to another section of the paper.

Other Species.—As indicated in the outstart, about a dozen different species were tried in the course of the investigation. Among these were several campanularian hydroids and two species of medusæ. The hydroids tested gave the same initial responses as those just described for *Tubularia*, but beyond that the results were likewise negative. In all cases the phase of cell-aggregation was essentially the same as in the former cases. The same was likewise true of the internal organization of the morula-like embryo, and in the perisarc formation, but beyond this there was no development.

Species of sertularian hydroids and also of *Halecium* were tested and gave exactly the same initial responses, including encystment of the embryonic mass which lived for a time but soon showed signs of disintegration and death. The reactions of the last species were the least satisfactory of any tested.

Medusæ.—Two species of medusæ were tried, though with hardly any hope of getting any regenerative responses. They were prepared just as had been the hydroids, strained through the bolting cloth and set aside after addition of fresh water. An examination of the dissociated cells showed about the same condition of the other preparations, and further inspection in about an hour showed a series of the most beautiful cell-aggregates found in any of the experiments. When it is recalled that Medusæ represent the most highly specialized group of Hydrozoa it will seem strange to find cells thus organized after having been dissociated in the manner indicated. In all my observations upon cœlenterate development I have seldom seen more typical blastula-like embryos than those under review. Unless one were actually aware of their source he could hardly have been convinced that they were not genuine embryos in process of development. However, so far as my experiments show the regenerative process does not go farther. Moreover, the organism thus formed is very short lived, and devoid of further significance so far as our problem is concerned.

Addenda and Discussion.

As mentioned in the introduction the immeditae occasion leading to the publication of this paper at this time after having been laid aside for four years was the appearance in the *Journal* of the Marine Biological Association, October, 1914, of a paper by DeMorgan and Drew, setting forth the results of similar experiments, all of which had given generally negative results. Moreover, certain of their conclusions seemed to leave a measure of doubt concerning the conclusiveness of certain of Wilson's experiments, and phases of their discussion involved assumptions which are at variance with those which my own work had rendered very convincing.

In the first place I desire to refer briefly to Wilson's methods and results with most of which my own are in accord. His experiments on *Eudendrium* seem to have been much more successful than my own, for which I am very glad, since it confirms with great certainty points which in my own experiments were incomplete, though sufficiently complete to warrant definite conclusions. In another point Wilson's work goes beyond my own, namely, in the admirable demonstration which his actual sections of various stages affords as to the precise features involved in the regenerative process at given times. Furthermore, the excellent series of drawings and photographs illustrating his results leave nothing to be desired in that respect, and I am purposely omitting any of my own, the only series of which not better covered are those relating to *Podocoryne*, and in these nothing essentially different occurs.

The work of DeMorgan and Drew covered experiments on two species of *Antennularia* and are restricted to these only. In order to consider certain of their views it may be well to first quote certain specific statements in their own words. "Our results largely bear out his (Wilson) contentions, though we were not successful in carrying the regenerative process as far as the production of new hydranths, and the histological structure of the restitution masses we obtained differed in many ways from that described in Wilson's paper. These differences are probably due to the fact that we experimented with other species of hydroids to those used by Wilson. The especial interest of

our investigations lies in the rather anomalous fact that we have not been successful in obtaining regeneration of the complete organism from the dissociated cells. In our experiments the restitution masses, by some rearrangement or metaplastic process taking place among their conglomerated cells, formed tissue aggregates histologically reduplicating the structure of the parent organism, but in a *quite irregular and apparently meaningless manner*."

Two features in this quotation call for brief consideration, that included in the first sentence, and that which I have italicized in the last. It will have been noted in the accounts given in the earlier sections of this paper that I have given a number of cases comprising the exact equivalent of the failure they mention. This point will be further noted in a later paragraph. To the second feature it is only necessary to state that in normal hydroid development the *entire process is often "quite irregular and apparently meaningless*," frequently more so than they found in the cases concerned. In a final paragraph the authors say: "Our experiments have resulted in the production of masses that are *certainly abnormal and pathological*, but nevertheless we would submit that the segregation and rearrangement of the cells after isolation, and the comparatively long duration of life of the tumor-like masses to which they give rise are facts of considerable theoretical interest."

In this quotation I have italicized the points to which it seems necessary to make some reference. It may be admitted that in some sense such restitution masses are *abnormal*, in that the very process by which their dissociation was brought about was presumably abnormal. But that the resulting restitution masses, involving as they have the regenerative potencies of the component cells, is abnormal I must seriously challenge. Again, the assumption that they are *pathological* I should emphatically doubt. The writer once submitted a series of preparations of embryological material to a well-known cytologist and received the (at that time) very disconcerting comment, "your preparations appear to have been made from pathological material." Yet from that very material I had been getting living embryos by the hundred! So in the present case to designate as pathological

cell aggregates which are producing right along perfectly normal and healthy polyps is to use a term whose significance implies the very opposite. It is admitted in the above citation that the tumor-like masses continued to live for a long time, as much as sixty days according to a preceding sentence, which shows a degree of vitality greater than that of colonies of the hydroid when placed in the aquarium. This fact of itself should prompt serious hesitation as to an assumption of a pathological condition.

It may throw some light upon the problem if attention is directed to conditions involved in the life history of many of these organisms. It is well known that many hydrozoa have alternating periods of activity and repose—growth, reproduction, etc., followed by corresponding periods of decline and more or less degeneration. In some these periodic alternations are correlated with seasonal changes in which temperature is an important factor. In others it is directly correlated with reproductive activities and has apparently little relation to season or temperature. What is of immediate importance in this connection is the fact of rather evident degenerative phases. For example, it is well known that in the spring, following the active reproductive period in several species of *Tubularia*, there is a marked degenerative phase, first evident in the casting off of the hydranths of almost the entire colony, then the gradual disintegration of the whole trophosome, till within a period of a few weeks it is difficult to find an entire and vigorous vegetative colony. An examination of the histological condition of the degenerative cœnosarc reveals the fact of positive decline marked by cytolytic conditions which might really be designated as pathologic for the time being. But even here a continued study would probably reveal the fact of its being associated with perfectly normal cyclic phases of life, being in fact phases of varying *physiological states* to which reference has already been made in an earlier connection. Similar facts of degeneration phases have also been described as associated with regenerative activity in hydroids. In experiments on *Tubularia* Stevens states in so many words: "The red granules seen in the circulation of regenerating pieces of *Tubularia* are derived from the disintegrating entodermal ridges, and are ejected by the young hydranth soon after

it emerges from the tube. They are waste material rather than formative substance" ('01, p. 414). The writer himself has made similar observations in several cases, and has demonstrated the regressive condition in the cœnosarc of hibernating specimens.

A most interesting study of a series of changes of apparenly-similar character has been made by Schultz, "Über Hungererscheinungen bei *Hydra fusca*" ('06), discussed under the larger topic of "Reductionen," under which are considered a series of marked phenomena observed in organisms of various grades of complexity, including *Planaria*, *Lumbricus*, *Æolosoma*, etc.

Similar experiments by Greeley ('03), on "Effects of Variations of Temperature on Animal Tissues," show essentially the same phenomena. Among these experiments some made on *Hydra* are especially pertinent in this connection. To quote, "It was at once observed that whenever a *Hydra* is exposed to a temperature of 4° to 6° C. the tentacles gradually become thicker and shorter, and finally are completely absorbed into the body. As the absorption goes on, the ectoderm and entoderm cells of the tentacles lose their individuality and form an undifferentiated mass of protoplasm, which is slowly resolved into the body of the *Hydra*. The tentacleless body of the *Hydra* becomes slowly resolved into a dense spherical mass of coagulated protoplasm, in which no distinction between individual cells can be made out, and remains in this condition as long as it is kept at a low temperature, but quickly forms tentacles and a double layer of cells again when it is returned to the temperature of the room" (p. 43).

Enough has now been said to show, I think, that only in some qualified sense can one use such terms as *irregular*, *meaningless*, *abnormal*, *pathological*, etc., in describing phenomena such as those involved in the experiments and results under review.

As a final note it may be stated that in my experiments no attempt was made to detach and isolate the several cell-aggregates such as was done by Wilson. Neither did I attempt to augment the masses by artificially bringing several masses into contact as he had done. Attention has already been directed to the fact that there was some evidence that from larger masses were derived larger polyps, and that below a certain minimum size there was no evidence of growth.

It need hardly be stated that my experiments add little essentially new to those of Wilson; yet they seem to afford valuable confirmation of some importance which may add to the conclusiveness of his admirably conceived and conducted research.

I desire also to express my gratification in the experiments of DeMorgan and Drew, which seem to me to have been admirably done and contribute to the value of the investigation as a whole.

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